A novel fibrinogen γ chain frameshift deletion (c.637delT) in a patient with hypodysfibrinogenemia associated with thrombosis

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Summary
Inherited fibrinogen (FG) disorders are rare and result in quantitative or qualitative FG deficiency. While the majority of patients with clinically relevant FG deficiencies demonstrate a bleeding phenotype, a subset of patients are at increased risk of thrombosis. Patients and methods: We report a 54-years old man presenting with a thrombophilic phenotype characterized by two episodes of unprovoked venous thrombosis and a deep vein thrombosis several weeks after myocardial infarction. Recently, he developed A. carotis communis thrombosis and died. Coagulation tests were done using standard procedures. FG genes were screened using direct sequencing. Effect on fibrin clot structure was analyzed by scanning electron microscopy (SEM) and FG chain polymerization was analysed using SDS-PAGE. Results: While thrombophilia testing was negative, we found a decreased concentration of clottable FG (126-148 mg/dl) compared to FG antigen (182-194 mg/dl of normal). The thrombin time was slightly prolonged, while aPTT and reptilase time were within the normal range. A novel deletion in FGG gene (c.637delT) resulting in a frameshift and the premature termination of the γ chain at amino acid position p.228 was identified. SDS-PAGE showed a time-shift in γ-γ and α-α cross linking. SEM showed no statistically significant differences between the patient’s and a healthy control’s fibrin clot structure. Conclusions: In addition to the reduction of FG concentration expected by the nature of the mutation also a functional defect (hypodysfibrinogenemia) was found. Moreover this mutation seems to increase the risk of thrombosis warranting long term anticoagulation possibly in a combination with antiplatelet drugs.

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Zusammenfassung
Fibrinogen (FG) is a coagulation factor building up the basic network of the final blood clot. Further important physiological functions of FG are platelet cross-linking as part of primary haemostasis and a contribution to blood viscosity (1). FG is the most abundant coagulation factor in the plasma reaching average concentration of 200–400 mg/dl and synthesized by hepatic parenchymal cells. (2).

The genes (FGA, FGB and FGG) encoding FG chains (Aα, Bβ and γ) are clustered on chromosome 4q32.1 (3) The 340 kDa large glycoprotein FG consists of two non-identical sets of Aα, Bβ- and γ-chains covalently linked by 29 disulfide bonds. The two chain sets build up the two outer D domains and one central E domain dominated by coiled-coil regions at the N-terminus of each chain. FG molecules are elongated 45 nm structures which get converted to fibrin by thrombin cleavage of two fibrinopeptides A and B from the Aα and Bβ-chains respectively on the central E domain. This initiates a series of lateral aggregation events resulting in the formation of protofibrils. Coagulation factor XIII (FXIII) interacts with FG in the plasma catalyzing the covalent cross links between α-α, γ-γ, α-γ of the lateral fibrin chain aggregates to give the blood clot biomechanical strength and resistance to fibrinolysis (4).

Hereditary FG disorders comprise of two classes of plasma based FG defects (1): - Type I, afibrinogenemia or hypofibrinogenemia, which has absent or low plasma FG antigen (Ag) levels (quantitative FG deficiencies).

- Type II, dysfibrinogenemia or hypodysfibrinogenemia, which shows normal or reduced Ag levels associated with disproportionately low functional activity (qualitative FG deficiency).

Patients with FG deficiencies show a spectrum of bleeding symptoms, thrombotic events and can occasionally be asymptomatic as well. A study compiled from the SSC Subcommittee meeting on Fibrinogen reported that in dysfibrinogenemia 55% of the patients had no clinical complications, 25% exhibited bleeding, and 20% showed thrombotic tendency, mainly venous (5).

Interestingly, the unpublished data of Casoni from Italy showed that approximately 20% of patients with afibrinogenemia experienced thromboembolic events too (1). Thromboembolism may occur either spontaneously or in association with FG substitution therapy. Here we report a rare case of a novel frameshift deletion in FGG gene (c.637delT) resulting in the premature termination of γ chain that is associated with an unexpected qualitative FG deficiency and a thrombophilic phenotype.

**Patient, material, methods**

**Coagulation and genetic analysis**

Thrombophilic markers and other coagulation parameters involving FG assays (functional (according to the Clauss method) and immunologic concentration) have been analysed using standard procedures.

The genomic DNA was extracted from peripheral blood after informed consent for genetic analysis. The exons and the flanking regions of FGA, FGB and FGG genes were sequenced directly using primers created by the Primer3 programme (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_cgi/) on a ABI3130 sequencer (6).

**Fibrin clotting test methods**

**SDS/PAGE analysis of γ-γ and α-α dimerisation**

For activation of blood clotting the citrated plasma sample was mixed with 0.2 U plasma purified α-thrombin (Sigma Aldrich, Germany) in the presence of 1 mmol/l calcium. Normal human pool plasma was used as control. After several time points (0 min, 5 min, 10 min, 30 min, 60 min, 90 min) the process was stopped by addition of 100 μl EDTA (50 mmol/l). Washed clots were dissolved in 15 μl PBS and 15 μl Laemmli Buffer (BioRad, Germany) and denatured for 8 min in 96°C. Protein bands were separated on a 10% Tris-Glycine-Gel (Bio-Rad) under denaturing conditions for 95 min with 140 Volt and visualized with Coomassie (BioSafe, Biorad, Germany) staining.

**REM-Untersuchung der Fibringerinnel zeigte keine statistisch signifikanten Unterschiede zwischen dem Patienten und einem gesunden Probanden. Schlussfolgerungen: Trotz eines zu erwartenden quantitativen Fibrinogenmangels aufgrund der Mutationsnatur (c.637delT) wurde ein qualitativer FG-Defekt (Hypodysfibrinogenämie) diagnostiziert, der ein hohes Risiko für thrombotische Ereignisse aufweist, sodass Patienten mit dieser Mutation eine Langzeit-Antikoagulation benötigen, möglicherweise in einer Kombination mit Antiaggreganzien.**

**SEM pictures**

To analyse the effect of the mutation on the fibrin structure the plasma (patient/control) was mixed with thrombin (0.2 U) and CaCl₂ (1 mmol/l) for activation of the clotting process. The clotting process was stopped after 60 min with 150 μl EDTA (50 mmol/l) and fibrin clots were fixed in 4 % glutaraldehyde (Sigma, Germany) and dehydrated with ethanol at increasing concentrations. Critical point drying (CPD), sample mounting, gold coating and viewing of the specimens in the SEM (scanning electron microscopy) followed for the structural analysis.

**Results**

This 54-years old man developed two episodes of unprovoked thrombosis (V. basilica 1986, V. jugularis 1993), and a deep vein thrombosis (DVT) several weeks after anterior wall myocardial infarction (MI) in 1994. Finally, he developed a complete thrombotic occlusion of the A. carotis communis and died after unsuccessful embolectomy several days after.

He was a smoker (20 cigarettes per day) and had dyslipidemia (elevated LDL/HDL...
cholesterol ratio and hypertriglyceridemia). His lipoprotein (a) level was normal. We found a decreased concentration of functional FG (126–148 mg/dl, normal range 180–355 mg/dl) compared to FG antigen (182–194 mg/dl, normal range 180–400 mg/dl). The thrombin time was slightly prolonged (27.6 s), while aPTT (24.6 s) and reptilase time (16.7 s) were within the normal range.

He showed no specific thrombophilic markers. The plasminogen activator inhibitor (PAI-1) polymorphism (4G4G) was found in homozygous form and had (2011) mildly elevated (57 ng/ml, normal range <47 ng/ml) levels of PAI-1. The repeated measurement of PAI-1 in 2013 revealed normal levels (31 ng/ml).

Our patient received oral anticoagulation (phenprocoumon) since 1994 after occurrence of deep venous thrombosis. At his own request (2013) oral anticoagulation was approximately one year prior to his death stopped and switched on antiplatelet therapy with acetylsalicylic acid (100 mg/d). Later on the dose was increased to 150 mg daily due partial response in platelet function analyzer (PFA100) test. No elevated D-dimers have been documented after discontinuation of phenprocoumon.

The mother of the patient also experienced multiple venous thrombosis and was for a long time under oral anticoagulation with phenprocoumon. No further information (also about the two children of the patient) was available.

**Genetic analysis**

Sequencing of FGG gene revealed a novel heterozygous 1 bp deletion in exon 6 (c.637delT, p.213Ser (TCT)) resulting in the premature termination (stop) of the γ chain at the amino acid position p.228 (exon 7). In addition, a homozygous γ 10034C>T (rs2066865) variant in FGG 3’ untranslated region (3’UTR) has been identified. No further mutations have been detected in FGA, FGB and FGG genes. The deletion of the Thymine causes a frameshift that creates a new cysteine downstream (▶Fig. 3) at the C-terminal. A new cysteine residue at the C-terminal end of the γ chain can have harmful consequences. This has been discussed more elaborately later.

The γ 3’UTR 10034C>T variant is known as a mild risk factor for venous thromboembolism (10).

**SDS-PAGE fibrin crosslinking analysis**

In the Coomassie gel picture we observed for the patient (▶Fig. 1B) a time shift in the formation of γ-γ dimers compared to the normal pool plasma (▶Fig. 1A). Full γ-γ dimerization is detected only after 90 min (▶Fig. 1B). The α-Polymers of the patient (▶Fig. 1B) seem to be not present up to 60 min of clotting but appear in full strength after 90 min unlike normal pool plasma where one can observe a gradual increase (▶Fig. 1A). The clot thickness...
Inherited fibrinogen (FG) disorders are in addition to the reduction of fibrinogen concentration expected by the nature of the mutation also a functional defect (hypodysfibrinogenemia) was found. The identified mutation might increase risk of venous and arterial thrombosis warranting long term anticoagulation possibly in a combination with antiplatelet drugs.

**What is known about this topic?**

1. Inherited fibrinogen (FG) disorders are rare and result in quantitative or and qualitative FG deficiency.
2. While the majority of patients with clinically relevant FG deficiencies demonstrate a bleeding phenotype, a subset of patients are at increased risk of thrombosis.

**What does this paper add?**

1. In addition to the reduction of fibrinogen concentration expected by the nature of the mutation also a functional defect (hypodysfibrinogenemia) was found.
2. The identified mutation might increase risk of venous and arterial thrombosis warranting long term anticoagulation possibly in a combination with antiplatelet drugs.

**Discussion**

Here we describe a patient with arterial and venous thrombotic events associated with hypodysfibrinogenemia and cardiovascular risk factors (dyslipidemia and smoking). The major question raising from this report is if the identified mutation (c.637delT) was one of the risk factors for thrombosis development. This patient was found to carry a deletion in the γ chain. Normally, we would expect that a heterozygous deletion would lead to the quantitative reduction of FG by 50%. This was not the case in our patient since discrepancies between clottable and immunological FG was observed for this patient and was confirmed by two independent laboratories. The deletion in this particular position (c.637delT) leads to a truncated protein (227 instead of 461 amino acids) i.e. partial deletion of the γ/γ′-chain of the FG. The frameshift induced by this deletion results in the creation of a cysteine residue at the C-terminal end of the γ chain (p.219Cys, Fig. 3). Two previously reported FG γ-chain heterozygous cysteine substitutions (IJmuiden BβArg14Cys and Nijmegen BβArg14Cys) have also been observed to result in an increased risk of thrombosis (11). The main reason attributed to this observation in these reports was that the mutated Cys variant most likely forms disulfide bonded higher molecular weight complexes with other prominent proteins like albumin. The presence of these complexes might cause thrombosis. Similarly, the Cys residue generated at the C-terminal end of the FG γ-chain due to the frameshift in our patient might also participate in similar disulfide linked complexes which in turn might explain the thrombotic phenotype of the patient.

Fibrin γ-γ dimerization was delayed for the patient compared with the healthy control i.e. the γ-γ dimers were observed only after 60 min while full cross linking was achieved only after 90 min suggesting slower crosslinking mediated by FXIII.

Normally the first γ-chain crosslinks between two fibrin molecules occurs within 5 to 10 minutes (12). Cross linking of the α-chains involves a number of glutamine and lysine residues and appears slower than γ-γ dimerization (12). In the normal pool plasma we observed α-chain crosslinking after 30 min which was not the case for the patient’s plasma in which the α-polymers appeared only after 90min. Since the γ chain region in question (i.e. the C-terminal region) is one of the putative regions suggested to be involved in FXIII-FG interaction, this mutation might have an impact on such interactions, thereby delaying the rates at which the cross links are formed. The SEM images of the fibrin clot does not support any significant difference in the thickness of the fibrin clots indicating that the mutation does not influence the end point physical characteristics of the clot.

**Conclusion**

We have identified a frameshift deletion in FGG gene c.637delT causing hypodysfibrinogenemia but not hypofibrinogenemia. This mutation is hypothesized to increase the risk of venous and arterial thrombosis warranting long term anticoagulation possibly in a combination with antiplatelet drugs (e.g. aspirin, clopidogrel).

**Conflict of interest**

The authors declare that they have no conflict of interest.

**References**


